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Formation of Nitric Oxide Myoglobin: Mechanisms of the Reaction with Various Reductants

SUMMARY—The concentration, temperature and pH dependences of the formation of nitric oxide myoglobin (NOMb) from metmyoglobin nitrite (MetMb · NO_2) were determined for nitrite and the reductants, ascorbic acid, cysteine, hydroquinone, nicotinamide adenine dinucleotide (NADH) and glyceraldehyde. The reaction for all reductants except glyceraldehyde involves the production of a nitroso-reductant intermediate which breaks down to release nitric oxide. The latter forms a nitric oxide metheme complex (Fe^{+++}) which is then reduced to the ferrous state (Fe^{++}). With cysteine and NADH there is a second pathway which probably involves the direct reduction of MetMb · NO_2. Ascorbate and hydroquinone form nitroso intermediates that are stabilized in alkali. The effects of oxygen, ethylenediaminetetraacetic acid and cytochrome *c* on the reaction were determined. Oxygen slows or inhibits the reaction, while the latter two have no effect on the reaction as studied.

INTRODUCTION

TO DATE, IMPROVEMENTS in cured meat color technology have been made on a largely empirical basis since the mechanisms of chromophore production in meat or meat products are unknown. It is known that the process is one of reduction of nitrous acid (from added nitrite) to nitric oxide and of metmyoglobin and methemoglobin from the ferric (met) to the ferrous state. But there are a number of reducing systems and/or reductants in meat which, in addition to or conjunction with added reductants, are capable of effecting the reduction.

Although isolated studies of the effects of various reductants and/or reducing systems have been made (Kelley *et al.*, 1957; Siedler *et al.*, 1959; Fox *et al.*, 1963; Walters *et al.*, 1965; Borys, 1965; Watts *et al.*, 1966), the accumulated data do not cover all conditions of pH, temperature and reactant concentrations encountered in cured meat production. The development of comprehensive data on the foregoing conditions for endogenous or added reductants can have a two-fold value, (1) by helping establish which reductants or systems are principally involved in meat curing, and (2) indicating the mechanism(s) of the reactions involved. Once these mechanisms are known it will be possible to determine the optimal conditions for obtaining maximal rates of color production conversions and stability.

We therefore undertook an *in vitro* study of the kinetics and thermodynamics of the formation of NOMb with the aim of producing coherent data on the pH, temperature and concentration dependences of the rate of nitric oxide heme pigment formation using reductants either normally added or endogenous to meat, or else potentially useful in the process. The data were used to develop equations that express the rate dependence of the reaction in terms of these various factors and reductants, and to determine the mechanisms of the reactions where possible.

EXPERIMENTAL

THE TECHNIQUES USED for the preparation of myoglobin have been described previously (Fox *et al.*, 1963). NADH was obtained from Nutritional Biochemicals Corporation; the rest of the chemicals were reagent grade. The nitric oxide came from the Matheson Company and the nitrogen was water-pumped prepurified grade.

The course of the reactions was followed by the change in absorption at 547.5nm as this was the wavelength of maximal absorption (β peak) in the visible portion of the spectrum as determined with a Cary Model 14 recording spectrophotometer calibrated to within $\pm$ 0.02nm. The Cary Model 14 or a Gilford attachment for a Beckman DU spectrophotometer was used to record the change in absorption; the Cary was used to record spectra. Temperature control was attained by means of a YSI thermoregulator with the thermistor probe either immersed in the cuvette or used as a surface probe fastened to the cuvette holder. The YSI unit controlled a Waco Refrigerated Visibility Bath. With this arrangement it was possible to control the cuvette to within plus or minus 0.05°C.

Reactions were run under three different conditions: (1) under nitrogen; (2) in open cuvettes without prior removal of oxygen; and (3) with air bubbling through the cuvette during the reaction. One centimeter cuvettes were fitted at the top with short sections of 6 mm tubing so that the cells might be sealed with size 000 serum bottle caps. Gases and solutions were introduced into the cuvettes through hypodermic needles inserted in the caps. Nitrogen gassing was conducted for a period of 10 min. Reagent solutions were made with nitrogen-gassed water.

NOMetMb was produced by bubbling nitric oxide through MetMb solutions after oxygen removal. A criterion of the conversion to NOMetMb was that the 575nm absorption maximum be higher than the 535nm absorption; lesser conversions gave poor results.

The reductants used in this study were ascorbate, cysteine, NADH, hydroquinone, and glyceraldehyde, chosen as being endogenous to meat or as containing functional groups corresponding to endogenous reductants. Table 1 summarizes the order of the dependences of the reaction rate on the concentration of the various reactants excepting glyceraldehyde. Table 2 lists a number of empirical equations for the various dependences on pH and temperature. With the exception of the reaction with NADH at pH 5.0

Table 1. Reaction rate on concentration.

Reductant	From metmyoglobin nitrite order of dependence, n				From nitric oxide myoglobin order of dependence, n			
			mM Nitrite					
	[R]	pH	<0.7	>0.7	[MMb]	[R]	pH	[MMb]
Ascorbate	0.50	−1.43[1]	1.0	0.41	0	1.0	0	1.0
Cysteine	0.48	−0.60	1.0	0.22	0	1.0	0	1.0
Hydroquinone	1.00	−1.2[1]	1.0	0.62	0	1.0	0	1.0
NADH	0.58	−1.19	~1.0		1.0[2]	1.0	0.1	1.0
					0[3]			

[1] 20°C.
[2] pH 4.5.
[3] pH 5.5–6.5.

and below, the reactions were all zero order with respect to the concentration of pigment.

To determine the order of dependence of the reaction on the concentrations of reductant and nitrite, these concentrations were varied and the effect on the zero order rate constant (k_0) observed. The dependence was fractional order with respect to the concentration of cysteine and NADH and it was found necessary to make use of the logarithmic form (2) of the basic equation (1):

$$(1)\quad y = a\,x^n$$
$$(2)\quad \log y = \log a + n \log x$$

The plots are shown in Fig. 1. The value for n of 0.481 for cysteine is not significantly different from 0.500, and indicates that the reaction rate is dependent upon the square root of the cysteine concentration. The figure of n = 0.578 for the NADH dependence does differ significantly from 0.500, however. Fig. 2 shows the reaction sequences involved, and is as follows:

Nitrous acid, in the form of the anhydride reacts with the reluctant to form a semi-stable intermediate, as proposed by Dahn *et al.* (1958, 1960), which may then react either to release free NO (reaction 4) or with another molecule of intermediate to reform the initial reactants (backward reaction, 3). Reaction 6, being the formation of the nitric oxide met-heme complex, is fast compared to the other reactions. Reaction 7 is a reduction step, and either it or reaction 4 is the rate-limiting step. Reaction 3 is the step which introduces the square root dependence on the reductant concentration into the rate expression, and if $k_3 > k_4$, the dependence will be exactly 0.5, i.e., cysteine and ascorbate. If $k_4 > k_3$ the reaction will be dependent directly upon the concentration of the reductant (n = 1.0), i.e., hydroquinone. It therefore follows that where $k_4 \cong k_3$ the dependence will be somewhere between 0.5 and 1.0, which is the case with NADH.

If $k_4 < k_7$ the overall rate expression will be zero order with respect to the pigment, but if $k_4 > k_7$ or if the reaction mechanism proceeds by some other pathway, for example, reaction 5, or reactions 8 and 9, the overall rate expression will be dependent upon the first power of the pigment concentration. The reaction sequence does not include the reduced form (ferrous) of the heme pigment, an omission based on the observation that at these pH values nitrite oxidizes the reduced heme pigments faster

Table 2. Coefficients of the equations describing the dependence of the rate of formation of nitric oxide myoglobin on pH and temperature.

Reductant	pH	t°C	Equation for log $10^2 k_0$[1,2]	ΔHa kcal/mole
Ascorbate	4.5	....	9.625 — 1804/T°K	8.26
	5.0	....	9.746 — 2050/T°K	9.38
	5.5	....	9.965 — 2323/T°K	10.63
	6.0	....	10.169 — 2593/T°K	11.87
	....	20°	9.907 — 1.43 pH	.....
	....	40°	9.805 — 1.32 pH	.....
Cysteine	4.5	....	16.212 — 4225/T°K	19.3
	....	20°	4.423 — .599 pH	.....
Hydroquinone	4.5	....	9.616 — 1902/T°K	8.71
	5.5	....	10.343 — 2549/T°K	11.67
	6.5	....	18.091 — 5092/T°K	23.30
NADH	....	20°	7.931 — 1.19 pH[3]	.....
			log $10^3 k_{1st}$[4]	
NADH	4.5	....	12.467 — 2900/T°K	13.28

[1] $k_0 = \mu M/min.$
[2] The standard deviation ranged from 2 to 10%; the average and modal S. D. was 5%.
[3] pH 5.0–6.5.
[4] $k_{1st} = min^{-1}$.

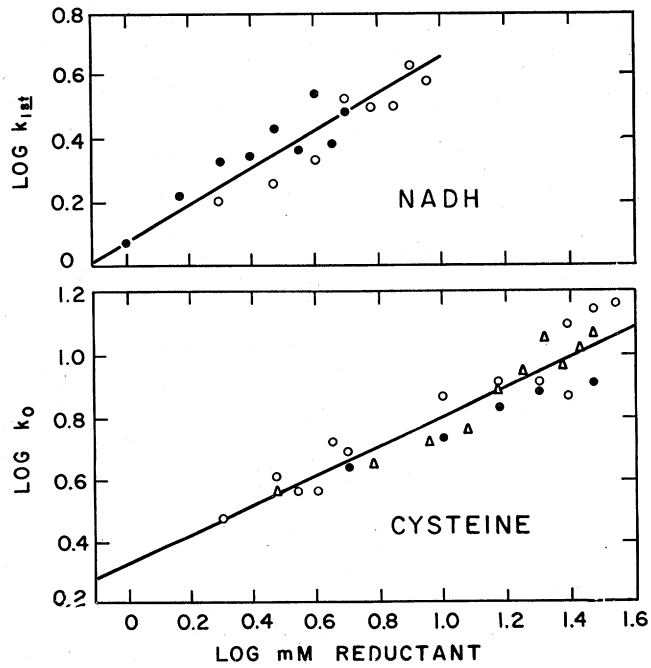


Fig. 1. Dependence of the rate constant for the formation of NOMb on the concentration of the reductants NADH and cysteine.

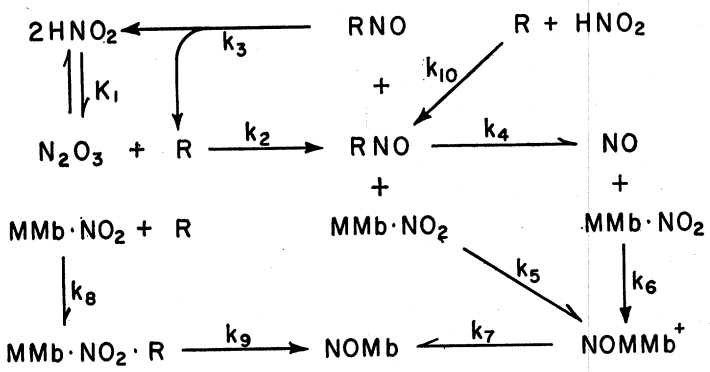


Fig. 2. Reaction sequences for the formation of NOMb from MetMb·NO₂.

than the nitric oxide heme pigments are formed, i.e., until the nitric oxide pigments are formed, the reduced heme pigments do not exist in solution in the presence of nitrite.

On the other hand nitric oxide combined with the oxidized pigment is probably stabilized against oxidation by oxygen and nitrite. The presence of the intermediate (NOMetMb) formed is observed spectrally during the course of the reaction from the position of the β peak (530–550 nm region), as follows. Since the maximum of NOMetMb appears at 532 nm, while the corresponding maximum for its reduction product NOMb lies at 547.5 nm, during the reaction a peak in this region appears first around 538–540 nm (indicating the presence of NOMetMb) and shifts towards a longer wavelength during the reaction until at the end of the reaction the final value of 547.5 nm is reached.

The variation with pH was fractional order with all four reductants as shown in Table 1. This fractional order may be the result of one or more of three causes: (1) the backward reaction 3 in Fig. 2 when the formation of the intermediate depends on pH; (2) two or more independent pathways to the same product with different pH dependences; and/or (3) two different forms of a semistable reaction intermediate, each form having a different heat of activation for breakdown. In the first case, the derived rate expression would be a precise mathematical derivation of the mechanism, but in the last two cases the expressions for the rate dependence are empirical. At the extremes of either pH or temperature the overall kinetics will be characteristic of one reaction mechanism or intermediate; at intermediate values the kinetics will reflect the behavior of all mechanisms and/or intermediates.

The reduction by ascorbate

The mechanism of this reaction has been discussed previously (Fox *et al.*, 1963) and was found to involve reactions 1, 2, 3, 4, 6 and 7. Reaction 4 was rate limiting, and, from the pH dependence, it was determined that the reaction was mainly dependent upon the unionized (acid) forms of the two acids, nitrous and ascorbic.

In the present study, the pH dependence was variable and somewhat less than 1.5 (1.43 at 20°C), in this case probably reflecting a variation in the stability of the nitroso-ascorbic acid intermediate. Since the heat of activation is the energy required to cleave the intermediate complex into an ascorbate radical and nitric oxide, its variation with pH is probably due to the ionization of the ascorbate carboxyl group, the ionized (alkaline) form being the more stable. It is important to note that the equations in Table 2 hold true only for the pH range 4.5 to 6.0. At 40° and pH 4.5 some cleavage of the protein-heme bond occurred producing some nitric oxide hemochrome; above pH 6.0 the reaction rate begins to increase with pH.

Reduction by cysteine

The basic assumption in solving the reaction sequence from the observed dependences is that the intermediates occur at constant concentration, "steady state," (Fox *et al.*, 1963). The desired rate expression which describes the principal reaction at pH 4.5 is the same for cysteine as for ascorbate, with different rate constants. The solution is obtained by assuming the reaction sequence includes reaction steps 1, 2, 3, 4, 6 and 7, with steady state concentrations of N_2O_3, AHNO, NO and NOMetMb. Reaction 4 is assumed to be rate limiting. The pH dependence $k_0 \sim [H]^{0.6}$, is very much different from what would be expected from a reaction involving only the unionized forms of the reactants.

Since the sulfhydryl group does not ionize appreciably in this pH range, it would be expected that the pH dependence would be due to the nitrous acid ionization and that k_0 would be proportional to $[H]^{1.0}$. From the low value of the order of dependence and the lack of variation with pH of the heat of activation, it is apparent that there is a second reaction which is an important pathway, particularly at the higher pH values. Since there were at least two mechanisms operative, we attempted to further elucidate the reaction mechanism by varying the concentration of the reactants at pH 6.5 where the effect of the reaction of the unionized forms would be minimal.

The rate of conversion to NOMb tended to be intermediate between zero and first order with respect to the pigment at higher pH values when the concentrations of cysteine and nitrite were 5m*M*. At nitrite concentrations above 5m*M* at pH 6.5, the reactions were all exactly first order with respect to pigment, the nitrite dependence disappeared completely, and the dependence of the first order rate constant on the cysteine concentration was very low ($n = 0.15$ in equation [2]).

The results indicate saturation of the system with both reductant and nitrite at the concentrations studied, and are consistent with a reaction sequence represented by reactions 8 and 9 in Fig. 2, showing the direct reduction by cysteine of $MetMb \cdot NO_2$. The heat of activation did not vary with pH, indicating that the scission of the S-N bond was not affected by ionizations in this pH range, regardless of the mechanism of the reaction. The value obtained is normal for the reduction reactions of cysteine, ca. 20 kcal/mole (Tarbell, 1961).

Reduction by hydroquinone

Hydroquinone was chosen as representative of the quinoid-type reductants that occur in animal tissue such as the tocopherols (E-Vitamins), K-vitamins, coenzyme Q and ubiquinone. Using the usual method of steady state analysis, it was determined that the reduction involved, in sequence, pathways 10, 4, 6 and 7, with reaction 4 again

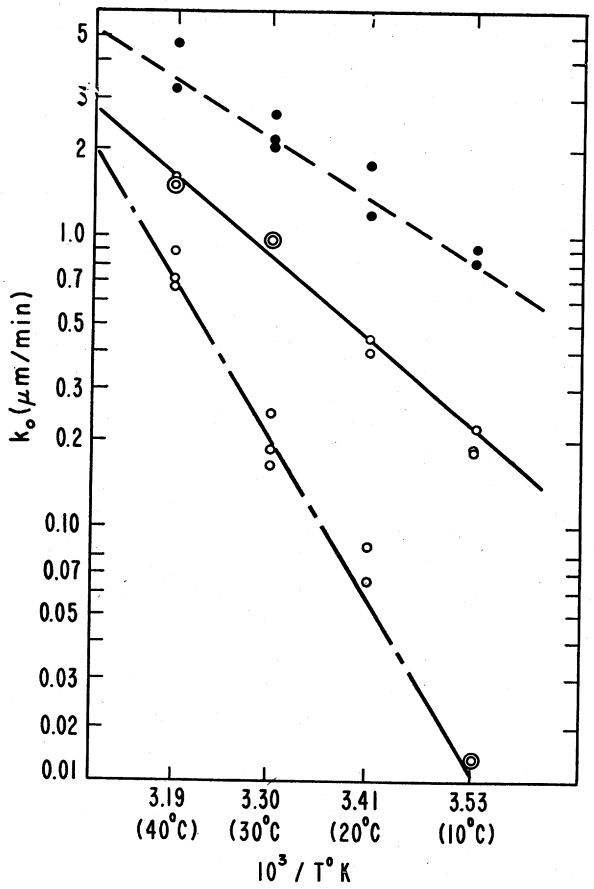
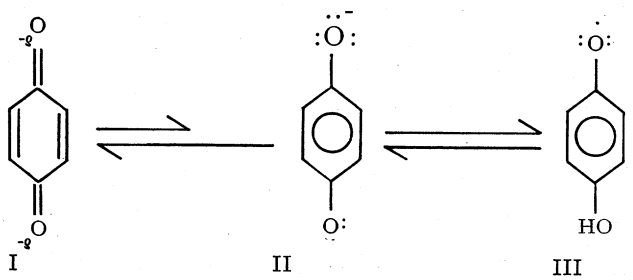


Fig. 3. Arrhenius plots for the formation of NOMb with hydroquinone as reductant. pH 4.5, - - -; pH 5.5, ——; pH 6.5, ——.

the limiting step. The release of NO and the formation of NOMetMb was attested to by the appearance of the β peak at 540 nm. The variation with temperature of the rate of the reaction increased sharply with decreasing hydrogen ion concentration, as shown in Fig. 3.

The heats of activation calculated from the Arrhenius plots are, in kcal/mole: pH 4.5, 8.7; pH 5.5, 11.7, and pH 6.5, 23.3. This increase is the result of the increased stability of the quinone intermediate at higher pH values as was also noted for the ascorbate intermediate. This increased stability in alkali is typical of quinoid structures containing oxygen and is the result of the delocalization of the charge of the ionized species in the π-bond structure of the semi-quinone (Gould, 1959).

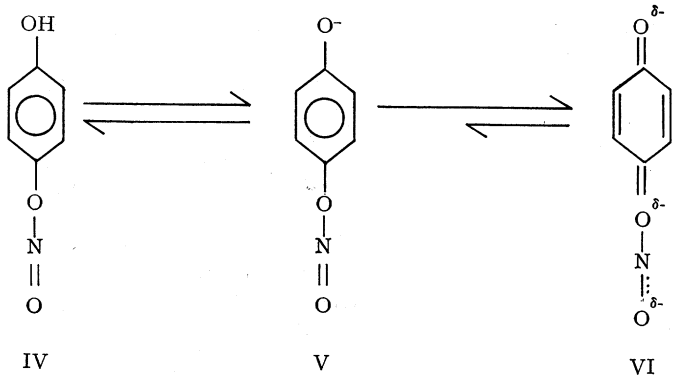
The theory of the resonance stability of the alkaline semi-quinones is represented in equation [3]:



I II III

In I the undissociated proton interferes with the participation of the p-orbital electrons of the oxygen in the resonant structure. In III, the structure is stabilized by the further inclusion of the two oxygen atoms in the π-bond system and the delocalization of the charge.

We therefore postulate the nitroso-semiquinone intermediate to be a structure containing both hydro-quinone and nitric oxide which may be formulated as follows:



IV V VI

Structure VI is the classical resonance representation of the stabilized structure and indicates the extensive delocalization of the π-bond electrons over the entire moelcule. It was not possible to assign specific heats of activation to either form of the intermediate because in the narrow range of pH 4.5 to 6 the reaction involved mixtures of both forms in unknown quantities.

The cleavage which releases nitric oxide is a one electron transfer presumably resulting in the formation of a semiquinone. We postulate that it is the ease of formation and stability of the semi-quinone as compared with the relative difficulty of formation of the radical products of the other reductants which accounts for the linear dependence of the rate constants on the concentration of hydroquinone and the fractional order dependences of ascorbate, cysteine, and NADH.

In a bimolecular reaction between two intermediate reductant-nitric oxide complexes, the bimolecular intermediate may either react to produce nitric oxide and the radical form of the reductant, or revert to the original electronic states of the reduced reductant and nitrous acid. The first reaction is in the forward direction. When the steady state analysis is made, the order of this reaction disappears and does not affect the order of the total reaction. The second reaction is in the backward direction and, if favored, introduces a fractional order dependence on the reductant. The quinone bimolecular intermediate will readily form the relatively stable radical product (first reaction) with the release of nitric oxide. In the case of the acid quinone, the formation of the meriquinone dimer may further stabilize the semi-quinoid product resulting from the release of nitric oxide, and contribute toward the ease of electron transfer.

In contrast, the other reductants, whose radical forms are not as fully stabilized as is the semiquinone structure, would tend to revert to their original state of reduction from the dimer rather than form the radical product, hence introducing a fractional order dependence.

Reduction by NADH

The total reaction sequence, as determined from the dependences of Table 1, involves reaction steps 1, 2, 3, 4,

6 and 7. At pH 4.5 the reaction is first order with respect to the pigment, but at pH 5.5 and higher values the conversion of pigment is zero order. The rate of the reaction at pH 4.5 must therefore be governed by a slow reaction after the involvement of the pigment. In the sequence of Fig. 2 it would be reaction 7, the reduction of NOMetMb. If the reduction of the last named pigment is the rate limiting step, then its rate should be equal to the rate of the reduction of MetMb·NO_2.

Table 3 summarizes the rate constants for the two reactions, and, as can be seen, at pH 4.5 the rates of the two reactions are the same. Comparing the turnover rates at higher pH values for the two reactions (0 order rate versus $k_{1st} \times 50$ μM pigment), it is seen that the rate of NOMetMb reduction is several magnitudes greater than the rate of MetMb·NO_2 reduction, hence the reduction of the latter is zero order. Such a situation did not prevail with the other reductants; at all pH values studied the rate of NOMetMb reduction was greater than the rate of nitric oxide production.

As in the case of cysteine the fractional order dependence of the NADH reduction is probably due to two or more different reaction mechanisms since the heat of activation did not vary with pH. However, we could not obtain data to support any further conclusions as to possible sequences, in part due to the formation of a reaction product between nitrite, NADH and heme pigment, which was not NOMb. The first reaction observed was the formation of NOMb followed by a reaction resulting in the formation of a stable heme pigment with a distinctive absorption spectrum, Fig. 4. The product is water-soluble and therefore presumably contains native protein, but we have not yet been able to convert it to any other recognizable heme pigment form.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH, TPNH) was tried as a reductant. Although a reddening of the solutions was observed, the NADPH denatured the protein with concomitant increase in turbidity.

Reduction by glyceraldehyde

The solutions of pigment, glyceraldehyde and nitrite did not show any spectral changes for periods up to 3 days at any pH between 4.5 to 6.5 and 20°C. It was necessary to raise the temperature to 40°C before any appreciable reactions were observed. At pH 4.5, the observed reaction was the production of nitrimetmyoglobin, the green heme pigment observed by Fox *et al.* (1964), while at pH 6.0 conversion to NOMb occurred overnight. The reaction

Table 3. Rates of reduction of metmyoglobin nitrite and nitric acid myoglobin by NADH.

pH	Pigment	Order	Rate constant	Turnover number [MMb]=50 μM
4.5	MMb·NO_2	1	0.187 min^{-1}	9.35
	NOMMb	1	0.211 min^{-1}	10.55
5.5	MMb·NO_2	0	0.71 μM/min	0.71
	NOMMb	1	0.224 min^{-1}	11.2
6.5	MMb·NO_2	0	0.083 μM/min	0.083
	NOMMb	1	0.182 min^{-1}	9.10

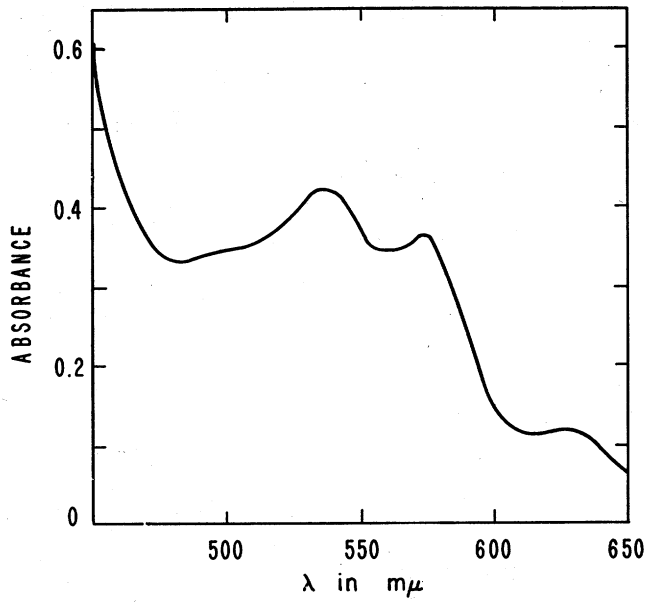


Fig. 4. Spectra of final product of reaction between NADH, nitrite and MetMb.

rate increased with increasing pH values above pH 6.0 and is probably a reaction similar to that observed for the other reductants above pH 6.5. At intermediate pH values, no reactions were observed up to 40°C.

Effect of nitrite

The concentration of nitrite was varied over a range of 0.05 to 50.0 mM at pH 4.5, 20°C in nitrogen, with each of the four reductants. The results were plotted on a log-log scale and the calculated values of n (Equation 2) are shown in Table 1. Two effects are noted: (1) the fractional dependence was observed only above 0.7 mM nitrite and (2) the fractional order varied with the reductant. The 0.7 mM nitrite corresponds to ca. 0.05 mM undissociated nitrous acid at pH 4.5, the same concentration as the pigment, and suggests an activation process involving the pigment. The fractional order dependences suggest multiple reactions between the reductant and the nitrite, with nitric oxide being but one of a number of products. In fact Evans *et al.* (1956) have found this to be true with the reaction between nitrite and NADH or ascorbate producing N_2O and N_2 as well as NO.

Reduction of NOMetMb

The reduction of NOMetMb was first order with respect to the pigment and with respect to the various reductants tested (ascorbate, cysteine, hydroquinone and NADH). The calculated rate constants were consistent with the observed rates of NOMb formation from MetMb·NO_2 and, except with NADH, did not vary with pH. The reduction of NOMetMb by NADH showed a low order dependence on pH (Table 1) which may be an effect of steric hindrance. NADH is a large molecule, and may not fit as well or as easily into the cleft in which the heme lies (Kendrew *et al.*, 1960; Perutz *et al.*, 1960). The pH dependence thus reflects changes in the protein conformation making the heme more accessible as the pH decreases.

Table 4. Effect of air on the reaction, $MMb \cdot NO_2 \rightarrow NOMb$.

Reductant	Gas	pH	Temp	k_0 μM/min	k_{1st} min^{-1}	Initial turnover rate μM/min
Asc	Air	4.5	20	....	0.315	13.3
	N_2	4.5	20	22.7 ± 2.5	....	22.7
	Air	4.5	30	....	0.649	23.1
	N_2	4.5	30	40.3 ± 3.6	....	40.3
	Air	4.5	40	....	1.23	33.0
	N_2	4.5	40	61.4 ± 0.3	....	61.4

Effect of oxygen

The reaction studied by Fox *et al.* (1963) took place in open cuvettes with no precautions to remove dissolved oxygen. All reactions discussed in this paper were run in closed absorption cells that had prepurified nitrogen bubbled through the solutions to remove oxygen. The rates of the latter reactions showed less variation and the rate constants were higher than in the previous study. In studying the effect of oxygen on the reaction, we bubbled air through the reacting solutions to maintain a constant level of oxygen in the solutions. The results are summarized in Table 4.

Two principal effects were noted; the reactions in air were first order with respect to pigment and the reaction rates were reduced, as indicated by turnover numbers in Table 4. This reduction in rate is probably a result of the relatively rapid oxidation of nitric oxide by oxygen. When the proposed nitroso-ascorbic acid intermediate breaks down to yield nitric oxide, in the absence of oxygen the only reactant available is the heme pigment.

With oxygen present, a competition between heme pigment and oxygen exists, and the relative amounts of nitric oxide consumed by the two pathways are dependent on the relative concentrations of pigment and oxygen, and the respective rate constants of the two reactions. With nitric oxide produced at a constant rate and the oxygen concentration constant, the observed fate of NOMb formation will depend on the concentration of MetMb available for complexing nitric oxide. Since this latter concentration decreases as the pigment is converted, the reaction becomes first order.

Effect of cytochrome c

Walters *et al.* (1965), studying the reduction of nitrite by mitochondria, found that ferrocytochrome *c* was oxidized by the system with the formation of nitric oxide. Since experience had shown us that our myoglobin preparations were sometimes contaminated with small amounts of cytochromes, we considered it advisable to determine what effect such contamination might have on the reaction. Our studies of the chromatography of myoglobin on carboxymethyl-cellulose showed that cytochrome *c* adhered firmly to the top of the column.

We placed myoglobin preparations on carboxymethyl cellulose columns under conditions that cause cytochrome *c* to adhere (0.05 *M* phosphate, pH 7.0), but found none, probably because it was completely dialyzed out of the solution in the two dialysis steps applied in preparing myoglobin. We therefore tested the effect of reduced cytochrome *c* on the reaction rate at 0.05 m*M*, 0.0005 m*M*, and zero concentration levels, but found no differences in the reaction rates. We therefore conclude that cytochrome *c* would have had no effect on the formation of NOMb in our reaction mixtures.

Effect of high pH

All the reductants exhibited a decreasing rate of $MetMb \cdot NO_2$ reduction with increasing pH up to pH 6.0 to 6.5. Above this pH the rate versus pH curve rose sharply. Even the reduction by glyceraldehyde showed an appreciable reaction rate above pH 7.0 at 20.0°C. From preliminary studies it appeared that the reaction mechanisms above pH 6.5 are different from those below pH 6.5 and that further study will be required to characterize them.

Effect of ethylenediamine tetraacetic acid (EDTA)

Siedler *et al.* (1959) and Weiss *et al.* (1953) reported that the addition of iron increased the formation of nitric oxide hemochrome; Pascal *et al.* (1957) reported that the oxidation of cysteine by peroxide was probably metal-catalyzed. We wished to know whether or not contamination was affecting our results, so we examined the effect of the addition of EDTA on our systems. The results were negative; the addition of EDTA neither changed the rate of the reaction, nor improved the precision of the measurements. This result is consistent with the observed negative pH dependence of the reactions, since metal catalyses are generally associated with positive rate-pH dependences (Pascal *et al.*, 1957).

DISCUSSION

OUR RESULTS EMPHASIZE some of the important aspects of the role performed by reductants in cured meat color production. Some of these aspects are (1) the effectiveness of any given reductant over the range of pH encountered in meat products; (2) its effectiveness in the elimination of oxygen and/or forming the nitric oxide pigment in the presence of oxygen; (3) the temperatures required to produce the nitric oxide heme pigments within reasonable time periods, and (4) the number of side reactions which take place that either use up the available reductants or produce nitrogen oxides no longer available for the production of cured meat pigments.

With regard to (1) and (3) it has been observed (Fox *et al.*, 1967) that cysteine is fully as effective as ascorbate in color production in frankfurter emulsions, after the oxygen has been eliminated and the emulsions are cooked. This is a happenstance of the pH and temperature dependences of cysteine and ascorbate. Thus, from the data of Table 2, it may be calculated that at pH 6.0 and 100°F (40°C) cysteine reduces $MetMb \cdot NO_2$ as fast as does ascorbate.

Sufficient oxidizable sulfhydryl groups do exist in meat to account for the observed rates; Hamm *et al.* (1965, 1966) found 17.5 m*M* (1965) and 25 m*M*-SH (1966) by amperometric titration. Assuming a 1:1 dilution in frankfurter emulsions, the sulfhydryl content of the added meat alone could account for the observed rates of nitric oxide hemochrome formation in frankfurter emulsions (Fox *et al.*, 1967). Sulfhydryl groups alone are only part of the available reductants in muscle tissue; Thomson *et al.*

(1962) and Regier *et al.* (1956) reported total reducible substance concentrations ranging from 50 to 100 mM.

Chromophore production itself is only one of a number of requirements. As Watts *et al.* (1966) and Fox *et al.* (1967) have shown, removal of oxygen, when present, is a vital part of cured meat color production. Ascorbate is more effective than cysteine in this respect, since the former reacts faster with oxygen and is capable of driving the reaction of $MetMb \cdot NO_2 \rightarrow NOMb$ to completion in the presence of oxygen. The latter reaction was accomplished only at the highest temperature studied (40°C) by cysteine.

Side reactions have been given little if any consideration to date, but may be of great importance. ten Cate (1962) obtained evidence of N_2O in the gases above curing pickles, showing that such side reactions do occur in practice. Our studies show that these reactions are particularly important at nitrite concentrations common to cured meat products (30-200 ppm, 0.7–5.0 m*M*) and that the relative rates vary with the reductant.

Since these side reactions produce nitrogen in oxidation states no longer available for the formation of nitric oxide, part of the overall effectiveness of any given reductant may depend upon the extent to which the reductant produces these compounds. As a general conclusion then, these studies suggest that no one reductant may be optimally effective in all cured meat products, but that consideration should be given to using different reductants or combinations thereof, depending on what kind of products are made and how they are made.

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Mention of commercial names does not imply endorsement by the U. S. Department of Agriculture.